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# Presentation of the Hydrophilic Domains of Hepatitis C Viral E2 Envelope Glycoprotein on Hepatitis B Surface Antigen Particles

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Subviral particles of hepatitis B virus have been used to present foreign epitopes. We attempted to present the hydrophilic domains of E2 envelope protein of hepatitis C virus (HCV) as a fusion protein with hepatitis B virus surface antigen (HBsAg). The five hydrophilic domains of HCV E2 antigen were inserted into HBsAg such that the inserted hydrophilic domains were presented on the outer surface of HBV subviral particles. In addition, a fusion encoding the hypervariable region (HVR) of E2 antigen was also made. Cell lysate and culture medium were analyzed for the synthesis and secretion of the fusion proteins by immunoprecipitation with polyclonal anti-HBsAg antibody using recombinant vaccinia virus system. The results showed that the fusion proteins containing these six E2 domains were made in the cell, but only two out of six fusion proteins were secreted into culture medium. Further, cesium chloride density gradient analysis and electron microscopy revealed that these fusions were secreted into culture media as particles. It will be of interest to test immunogenicity of the HBsAg fusion particles containing the HCV E2 domains in animal model. 

**KEY WORDS:** hybrid particles, HCV, HBsAg

## INTRODUCTION

Hepatitis C virus (HCV) is the major etiologic agent of post transfusion non-A, non-B hepatitis [Choo et al., 1989]. HCV infection progresses frequently into malignant liver disease, such as cirrhosis and hepatocellular carcinoma [Sakamoto et al., 1988]. Recent molecular cloning of HCV led not only to the identification of HCV as a major causative agent for post-transfusion non-A, non-B hepatitis but also to the development of a blood screening assay [Kuo et al., 1989]. However, no vaccine is available yet to prevent community-acquired transmission [Alter et al., 1992].

Molecular cloning and sequencing of the viral genome revealed that (i) it is a positive stranded RNA virus with a genome size of 10 kilo bases and has one large open reading frame that encodes a polyprotein of 3,011 amino acids [Houghton et al., 1991], and (ii) it has some structural homology with flaviviruses and pestiviruses [Miller et al., 1990]. One of the hallmarks of HCV infection is a high incidence of chronic infection. It has been reported that more than 60% of infections result in chronic infection [Alter et al., 1992]. Thus it is urgent to develop vaccine to eradicate HCV infection and HCV-associated liver diseases.

We were interested in the expression of the putative E2 envelope glycoprotein of HCV, since E2 glycoprotein of some flaviviruses and pestiviruses have been shown to confer protection against infection [Konishi et al., 1992; Schlessinger et al., 1987; Zhang et al., 1988]. The putative envelope glycoproteins of HCV, E1 and E2 antigens, were found as complex forms in the microsomal fractions of mammalian cells [Dubuisson et al., 1994; Ralston et al., 1993]. However, no complexes were secreted into culture medium as subviral particles. Since particulate forms of antigen are reported to be better immunogens, we chose to present potential epitopes of HCV E2 antigen on the hepatitis B surface antigen (HBsAg) particles. Surface antigen of hepatitis B virus (HBsAg) has been used to present foreign epitopes as hybrid particles, since HBsAg is secreted into culture media as particles [Delpeyroux et al., 1986; Michel et al., 1988]. We report the synthesis and secretion of the HBsAg particles containing the hydrophilic domains of HCV E2 antigen.

## MATERIALS AND METHODS

### Cells and Vaccinia Virus

BHK-21 cells and HeLa cells were used for transient expression as described previously [Fuerst et al., 1986]. Stocks of vTF7-3, a recombinant vaccinia virus expressing T7 RNA polymerase, were grown in BSC-40 cells and titers of infectious progeny were determined by plaque assay on BSC-40 cells.

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TABLE I. A List of Oligonucleotide Primers Used in This Study

| Clones | Primer | Sequence (5′ to 3′)[a] |
|---|---|---|
| HBsAg | PBsAG | AAAAAGGATCC**ATGGAGAACACAACATCA** |
| HBsAg | PSALsAg | AAAAAGTCGAC**TCAAATGTATACCCAAAGACA** |
| HVR | PBGSHVR5 | **TGTCCTCTACTTCCAGGA**AGATCT*GCCACCTACACAACAGGG* |
| HVR | PRSHVR5 | **CGTGCTGGTGGTTGATGT**GAATTC*GACTCTCTGAGACGCCCC* |
| HP1 | PBGSHP1 | AAAAAAGATCT*ACGACCGTGACGATGGGG* |
| HP1 | PRSHP1 | AAAAAGAATTC*GGAGTCATTGCAGTTCAG* |
| HP2 | PBGSHP2 | AAAAAAGATCT*TACGCTGAGAATGGCAGC* |
| HP2 | PRSHP2 | AAAAAGAATTC*CCGTCGGGGCGCGTAGTG* |
| HP3 | PBGSHP3 | AAAAAAGATCT*GATCGGTCCGGCGCTCCT* |
| HP3 | PRSHP3 | AAAAAGAATTC*CCGCGTGTTGTTGAGGAG* |
| HP4 | PBGSHP4 | AAAAAAGATCT*ACGGACTGCTTCCGGAAG* |
| HP4 | PRSHP4 | AAAAAGAATTC*GTAGTGCCAGAGCCTGTA* |
| HP5 | PBGSHP5 | AAAAAAGATCT*GAGCACAGGCTCATGGCT* |
| HP5 | PRSHP5 | AAAAAGAATTC*ACTGAGCTCTGATCTATC* |

[a]The restriction sites included in primers are underlined: BamHI (GGATCC), SalI (GTCGAC), BglII (AGATCT), and EcoRI (GAATTC). Nucleotide sequences derived from HBsAg and HCV E2 antigen domains are indicated in bold or in italic, respectively.

### Recombinant DNAs

The DNA fragment encoding HBsAg ORF was obtained by PCR of HBV clone (adr type) using primer PBsAg and PSALsAg (Table I), then cloned into pTM2 vector, pTM2(HBsAg). The pTM2 vector was derived from pTM1 [Fuerst et al., 1986], in which the Nco I site was destroyed by Nco I digestion, filling-in using Klenow polymerase and then religation.

The cDNAs of HCV obtained from a Korean patient were used for cloning of HCV E2 domains [Cho et al., 1995; Ryu et al., 1995]. pTM2(HBsAg/HVR) was constructed by several steps and contained the HBsAg ORF (open reading frame) with the insertion of E2 HVR domain at amino acid position 113 of HBsAg. To facilitate other insertions, the HVR domain of pTM2(HBsAg/HVR) plasmid was designed to contain flanking Bg1 II site at the N-terminus and EcoR I site at the C-terminus. Consequently, two amino acids were included at the junctions between HBsAg and the E2 insertions: Bg1 II site (AGATCT, Arg and Ser) and EcoR I site (GAATTC, Glu and Phe). Next, five insertions were made into the Bg1 II and the EcoR I sites of pTM2(HBsAg/HVR) by substitution. To make pTM2(HBsAg/HP1), the DNA fragment encoding HP1 domain of E2 antigen was obtained by PCR using primer PBGSHP1 and PRSHP1 (Table I), then digested with Bg1 II and EcoR I, and then cloned into the Bg1 II and EcoR I site of pTM2(HBsAg/HVR). Similarly, the insertions of HP2, HP3, HP4, and HP5 were made using appropriate primers, as indicated in Table I.

### Transient Expression by Vaccinia Virus

Briefly, subconfluent monolayers of cells in 35-mm dishes were infected with vTF7-3 (m.o.i. = 10) in serum-free MEM for 30–60 min. After removal of the inoculum, cells were transfected with a mixture consisting of 5 mg of pTM2 plasmid DNA and 15 mg of by Lipofectamine (BRL) in 0.5 ml of DMEM. After 2 hr at 37°C, the transfection mixture was replaced with 0.5 ml of L-methionine-deficient DMEM containing 1/40 of the normal concentration of methionine and 60 μCi of L-[$S^{35}$]-methionine (Amersham) per ml.

### Immunoprecipitation

After labeling for 4 hr, cells were washed with ice-cold PBS (phosphate-buffered saline), lysed with RIPA buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 20 mM EDTA, 0.1% SDS, 1.0% sodium deoxycholate, 1.0% Triton X-100). Samples were vortexed and clarified by centrifugation at 13,000 *g* for 10 min. Portions of each lysate were incubated with 10 ml of polyclonal goat anti-HBsAg (Dako, Carpinteria, CA) for 1 hr. The immune complexes were collected by using Protein-G sepharose (Sigma, St. Louis, MO) as described previously [Ryu et al., 1993]. Immunoprecipitates were analyzed by SDS-PAGE. After the treatment for fluorography, gels were dried and exposed to X-ray film (Kodak, Rochester, NY). To examine the secretion of HBsAg fusion proteins, culture media were harvested and immunoprecipitated in RIPA buffer as above.

### Enzyme-Linked Immunosorbent Assay (EIA)

Expression of HBsAg was also quantitated by EIA. Microtiter plates were coated with 100 μl of a 20 μg/ml of guinea pig polyclonal anti-HBs antibody in PBS for 16 hr at 4°C and washed with 0.5% Tween 20 in PBS. The plates were blocked with the same solution containing 3% bovine serum albumin and then, incubated with 100 μl of cell lysate or culture medium and 25 μl of various dilution of peroxidase-labeled anti-HBs monoclonal antibody. Absorbances were measured at 492 nm by Titertek Multiscan Plus (Flow Lab, Research Triangle Park, NC).

### Cesium Chloride Density Gradient Analysis

Culture media were collected and adjusted to 50 mM Tris-HCl, pH 7.5. CsCl was added to the concentration of 20%, and centrifugation was carried out at 4°C for 40 hr at 38,000 rpm in a Beckman SW-41 rotor. Twenty fractions were collected from the top by Auto Densi-

Flow IIC (Buchler,) and the amounts of HBsAg fusion antigens were measured by EIA. The density of each fraction was determined by a refractometer (Fisher, Springfield, NJ).

### Electron Microscopy

Fractions from CsCl gradient were examined by electron microscopy. Particles were visualized after being negatively stained with 2% uranyl acetate, as described previously [Ryu et al., 1992]. Micrography were taken at 144,000 magnification.

## RESULTS

The synthesis and cellular processing of the putative E1 and E2 envelope glycoprotein of HCV have been studied in mammalian cells [Dubuisson et al., 1994; Matsuura et al., 1994; Selby et al., 1994]. They were not secreted into culture medium as particles. Rather, their processing was blocked at endoplasmic reticulum. Previously, it has been known that the particulate forms of antigens are better immunogens for vaccination. To make the particulate form of E2 antigen, it was decided to make fusions between hepatitis B virus surface antigen (HBsAg) and HCV E2 antigen.

Previously, others employed hepatitis B viral surface antigen to present a well-defined B-cell epitope [Delpeyroux et al., 1986; Michel et al., 1988]. However, the principal neutralization determinant(s) of HCV still remains to be identified. Thus, we decided to make insertions of the hydrophilic domains of HCV E2 antigen (Fig. 1A and B), because (i) E2 glycoproteins of some flaviviruses and pestiviruses have been shown to confer protection against infection [Konishi et al., 1992; Schlessinger et al., 1987], and (ii) hydrophilic domains are likely to be presented on the outer surface of the particles. The hydropathic profile of E2 envelope region revealed five hydrophilic domains (Fig. 1A). The five hydrophilic domains were inserted at amino acid position 113 of HBsAg such that the inserted E2 domains were exposed on the outer surface of the fusion particles [Delpeyroux et al., 1990] (Fig. 1C). Besides, recent evidence suggests that the neutralization epitope may correspond to the hypervariable region (HVR) of E2 protein [Choo et al., 1995; Kato et al., 1994; Weiner et al., 1992]. Thus, along with the hydrophilic domains, we also constructed a fusion protein containing the E2 HVR domain with a size of 28 amino acids (Fig. 1B).

### Synthesis and Secretion of the Hybrid Antigens

To examine the expression and secretion of the HCV-HBsAg fusion proteins, we employed recombinant vaccinia virus expression system [Fuerst et al., 1986]. HeLa cells were infected by T7 polymerase-producing vaccinia virus, vTF7-3 and then transfected with the recombinant pTM2 plasmids, as described in Materials and Methods. The synthesis and secretion of the fusion antigens were then examined by immunoprecipitation of cell lysates and culture media with polyclonal anti-HBsAg antibody (Fig. 2).

Two bands (24 kDa, the nonglycosylated and 27 kDa, the glycosylated forms of HBsAg) were detected in cell lysate from the wild type HBsAg clone, pTM2(HBsAg) (Fig.2A, lane S), as described previously [Delpeyroux et al., 1990]. Similarly, two HBsAgs containing pre-S2 domain (33 kDa and 36 kDa) were detected in cells transfected with pTM2(Pre-S2) (Fig. 2, lane pre-S). The six hybrid antigens containing HCV E2 domains were detected between 29 kDa and 46 kDa size marker (Fig. 2, lane HVR and HP1 to HP5). The differences among the apparent molecular sizes of the various HBsAg proteins carrying insertions were in good agreement with the lengths of the amino acid sequences encoded by the inserts. In summary, the fusion proteins were synthesized in the cell as predicted from the constructs.

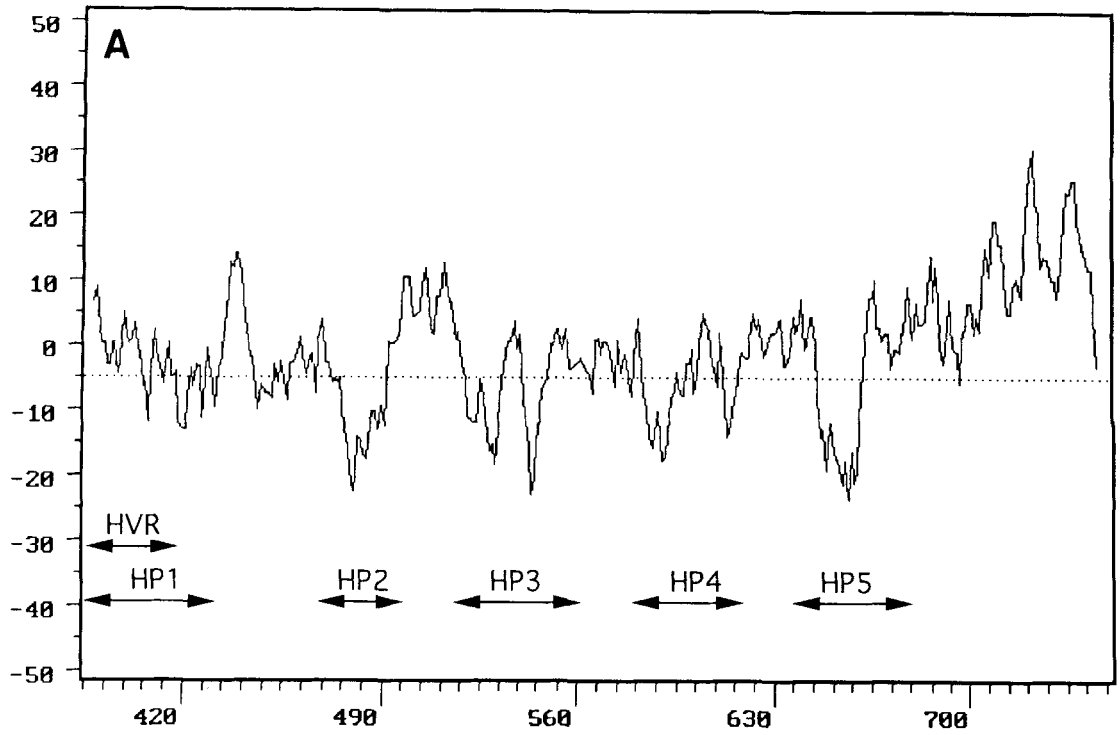
We also examined the secretion of the fusion proteins by immunoprecipitation of culture medium (Fig. 2B). As expected, the wild-type HBsAg was detected in culture medium (Fig. 2B, lane S). Very little amount of pre-S2 HBsAg was detected (Fig. 2B, lane pre-S). Although the six hybrid antigens were synthesized in the cell, only two hybrid antigens, HBsAg/HVR and HBsAg/HP3, were detected in culture medium (Fig. 2B, lane HVR and HP3). Interestingly, the size of secreted forms of HBsAg containing HP3 was about 6 kDa larger than that of cell lysate (Fig. 2, lane HP3 of panel A vs. lane HP3 of panel B), probably due to the glycosylation of the inserted HP3 domain. Consistently, two glycosylation consensus sites were found at 532–534 (Asn-Glu-Thr) and 540–542 (Asn-Asn-Thr) of the HCV-L2 isolate [Cho et al., 1995]. The glycosylation of the inserted domain suggested that the inserted HP3 domain may possess proper conformation. In addition to HP3, a fusion containing 28 amino acids HVR domain was also secreted into culture medium (Fig. 2B, lane HVR).

### Quantitation by EIA

The amount of the HBsAg hybrid proteins made in the cells and culture medium was also quantitated by EIA with polyclonal anti-HBsAg antibodies, as described in Material and Methods. As shown in Figure 3, we found a good correlation between the results of immunoprecipitation and those of EIA. In fact, the HBsAg/HVR particles were secreted into culture medium as efficiently as wild-type HBsAg. However, compared to HBsAg/HVR, HBsAg/HP3 particles were detected approximately 20-fold less in culture medium by EIA (Fig. 3), although comparable amounts of the hybrid particles were measured by immunoprecipitation (Fig. 2B). This discrepancy between the EIA results and immunoprecipitation could be due to the disruption of B-cell epitope of HBsAg by the insertion of HP3 domain.

### Evidence of the Fusion Antigen as Particles

Next, to demonstrate that the secreted fusion antigens were subviral particles, equilibrium density gradient analysis was carried out on cesium chloride. Culture media from cells transfected by pTM2(HBsAg/HVR) were subjected to equilibrium density gradient on cesium chloride as described in Materials and Methods (Fig. 4A). Twenty fractions were collected and the




**B** E2 domains Amino acid sequences

| | | |
|---|---|---|
| HVR | (28) | TTVTMGGTVARTTYGFTGLFRPGASQKI |
| HP1 | (49) | TTVTMGGTVARTTYGFTGLFRPGASQKIQLINTNGSWHINRTALNCNDS |
| HP2 | (20) | YAENGSSDQRPYCWHYAPRR |
| HP3 | (24) | DRSGAPTYSWGENETDVLLLNNTR |
| HP4 | (36) | TDCFRKHPEATYTKCGSGPWLTPRCLVDYPYRLWHY |
| HP5 | (27) | EHRLIAACNWTRGERCNLEDRDRSELS |

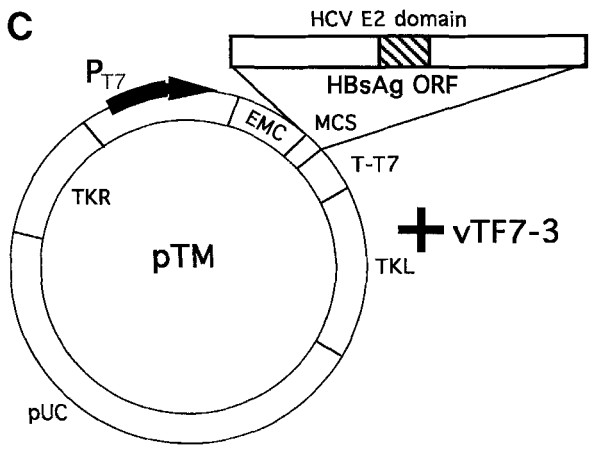



Fig. 1. Plasmid map of pTM2(HBsAg) fusion constructs used for the synthesis of the HBsAg hybrid particles **A:** Hydropathic profile of E2 ORF (open reading frame, from amino acid 384 to amino acid 750) is presented. The five hydrophilic domains and the hypervariable region (HVR) of E2 were indicated by arrows. **B:** The amino acid sequences encoded in the six domains of E2 ORF. Each insertion included the domains as follows: HVR (384–411), HP1 (384–432), HP2 (474–493), HP3 (520–543), HP4 (583–618), and HP5 (637–663). The number of amino acids encoded in each insert is indicated in parenthesis. The consensus glycosylation sites in the HP3 domains and cysteine residue are underlined. **C:** Plasmid map of pTM2(HBsAg) fusion constructs used for transient expression of the HBsAg fusion proteins. The HCV E2 domains were inserted at amino acid position 113 of HBsAg as indicated by hatched box. The internal ribosome entry site derived from encephalomyocarditis virus is indicated as EMC [Jang et al., 1989].

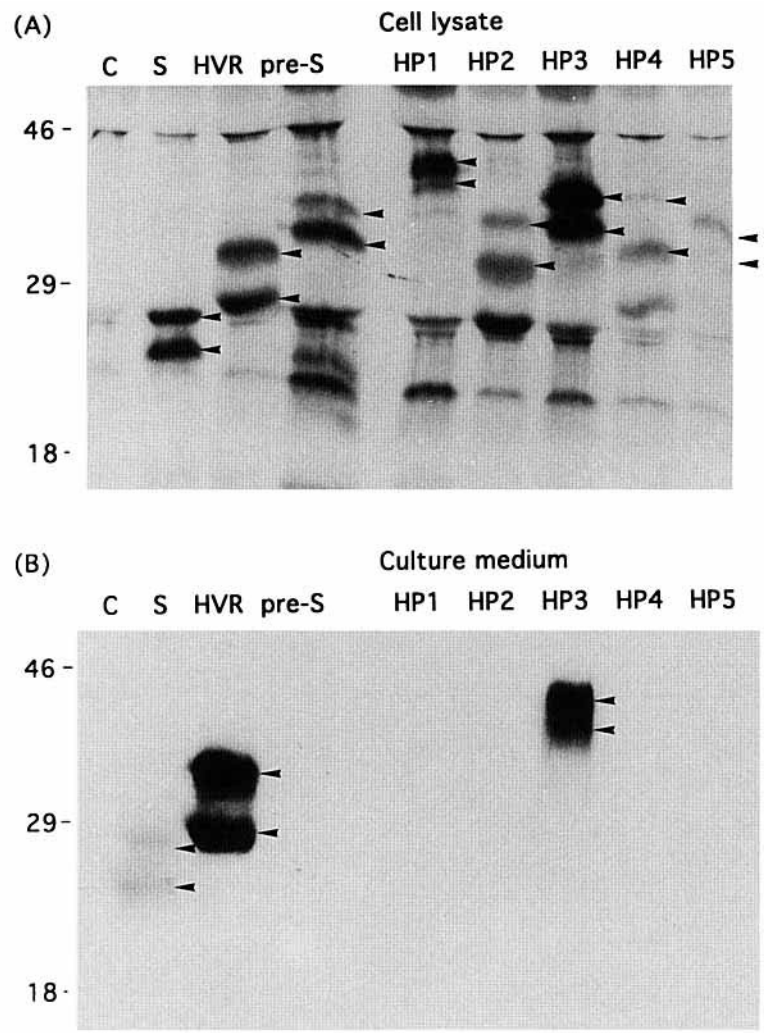



Fig. 2. Immunoprecipitation of the HBsAg fusion proteins. HeLa cells were infected by vTF7-3 and then transfected by various pTM2(HBsAg) fusion plasmids. Immunoprecipitation of cell lysate **(A)** and culture media **(B)** were carried out using goat anti-HBsAg antibody, as described in Materials and Methods. Each lane represents samples derived from cells transfected by as follows: Lane C, pTM2; lane S, pTM2(HBsAg); lane HVR, pTM2(HBsAg/HVR); lane pre-S, pTM2(pre-S2); lane HP1, pTM2(HBsAg/HP1); lane HP2, pTM2(HBsAg/HP2); lane HP3, pTM2(HBsAg/HP3); lane HP4, pTM2(HBsAg/HP4); lane HP5, pTM2(HBsAg/HP5).

amount of HBsAg was measured by EIA as described earlier. The density of the fusion particle was 1.20 g per $cm^3$, a density similar to that of HBsAg particles derived from HBV-infected human sera. Essentially, the same result was obtained from pTM2(HBsAg/HP3)-transfected cells (data not shown). These data strongly indicated that the HBsAg fusions were secreted as particulate forms rather than free antigen forms. The peak fraction from CsCl gradient was subjected to electron microscopic examination. Electron microscopy revealed the spherical morphology of the fusion particles (Fig. 4B). It appeared that the size of the fusion particles was slightly larger than the wild-type subviral particles: a mean diameter of both HBsAg/HVR and HBsAg/HP3 was approximately 30 ± 3 nm, whereas that of the wild-type subviral particles was 27 ± 3 nm.

## DISCUSSION

Recently, cellular processing of HCV polyprotein has been studied extensively [Dubuisson et al., 1994; Matsuura et al., 1994; Selby et al., 1994]. A complex form of two putative envelope proteins, E1/E2, was identified, although no secretion of E1/E2 glycoprotein complex was found. To obtain particulate forms of the viral envelope proteins, we chose to make hybrid with hepatitis B virus surface antigen (HBsAg), since (i) HBsAg is secreted into culture medium as particles, and (ii) HBsAg is a good carrier for presentation of foreign epitopes, since it could elicit a wide spectrum of immune responses, including induction of neutralizing antibodies, proliferative T-cell response, and cytolytic activities in immunized animals [Jin et al., 1988; Schlienger et al., 1992]. We report the synthesis and secretion of the HBsAg hybrid particles encoding hydrophilic domains of HCV E2 antigen.

We found that two out of six HBsAg hybrid proteins were secreted into the culture medium (Fig. 2B). At this point, we do not understand why the cellular processings of some of the fusion proteins were blocked in the cells.

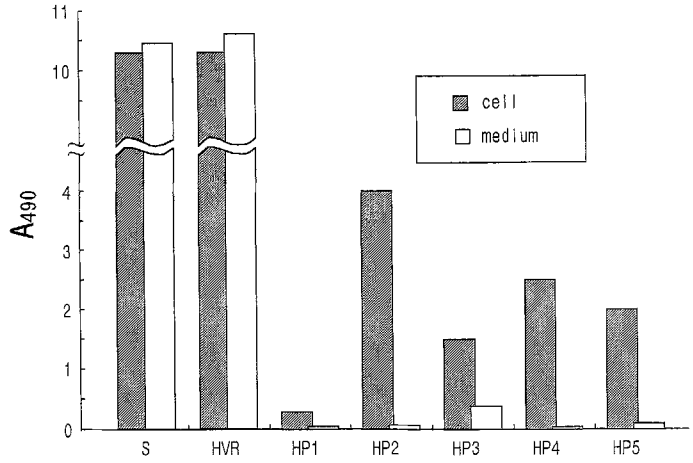



Fig. 3. Quantitation of the HBsAg fusion antigen in cells and culture medium by EIA. The positive control represents 20 ng/ml of yeast-derived HBsAg. Absorbances at 490 nm were measured. Each lane represents samples derived from cells transfected by as follows: Lane S, pTM2(HBsAg); lane HVR, pTM2(HBsAg/HVR); lane pre-S, pTM2(pre-S2); lane HP1, pTM2(HBsAg/HP1); lane HP2, pTM2(HBsAg/HP2); lane HP3, pTM2(HBsAg/HP3); lane HP4, pTM2(HBsAg/HP4); lane HP5, pTM2(HBsAg/HP5).

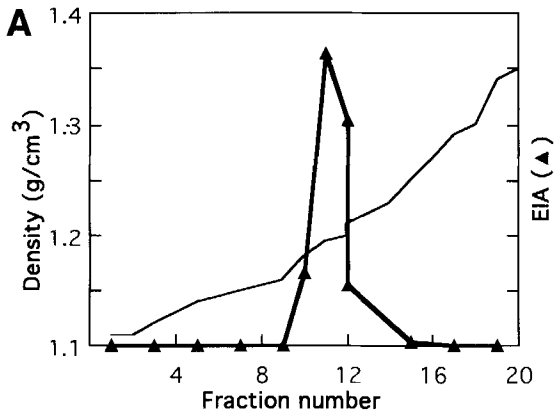
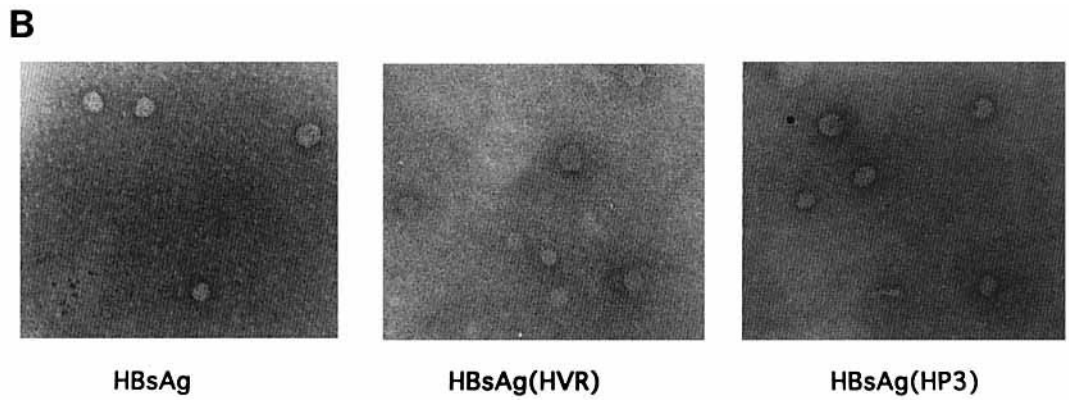



Fig. 4. Evidence that the HBsAg fusion proteins are secreted as particles in culture medium. **A:** Cesium chloride banding of the secreted fusion particles. The culture media were collected and centrifuged to equilibrium as described in Materials and Methods on cesium chloride gradient. Twenty fractions were collected and assayed for HBsAg by EIA. **B:** Electron microscopy of the secreted fusion particles stained with 2% uranyl acetate. Magnification, 144,000.




It is possible that the insertions were either too large or too hydrophilic to be compatible with HBsAg processing. In fact, only small insertions (e.g., 11 amino acids) were allowed previously at amino acid position 113 of HBsAg [Delpeyroux et al., 1990]. Interestingly, close examination of the insert revealed that no cysteine residue was encoded in the insert of two hybrid particle (HBsAg/HVR and HBsAg/HP3) that were secreted in culture medium, while at least one or more cysteine residues were included in the insert of other four hybrids (Fig. 1B). It is possible that cysteine residues in the insert may form disulfide bonds with one of cysteine residues of HBsAg, in turns disrupting proper processing. We are in the process of clarifying this issue by substituting cysteine of the insert with serine.

Generation of the recombinant HCV-HBsAg particles

can serve several purposes. First, the HBsAg fusion particles can be used to look for neutralization determinants in convalescent sera [Farci et al., 1994]. If the inserted E2 domains are presented properly, they might be recognized by neutralizing antibodies present in convalescent sera. Alternatively, the HBsAg fusion particles can be used to identify the neutralization epitopes after immunization of the hybrid particles encoding the potential neutralizing epitopes [Michel et al., 1988].

Recently, Choo et al [1994] reported successful vaccination of chimpanzees after immunization of mammalian cell-derived E1/E2 antigen complex. Thus, it will be of interest to test whether immunization of the HCV-HBsAg hybrid particles could elicit neutralizing antibody to protect HCV infection of chimpanzee.

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